THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 263, No. 30, Issue of October 25, pp. 15513-15520, 1988
Printed in U.S.A.

Increase of the DNA Strand Assimilation Activity of recA Protein by Removal of the C Terminus and Structure-Function Studies of the Resulting Protein Fragment*

(Received for publication, March 2, 1988)

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A proteolytic fragment of recA protein, missing about 15% of the protein at the C terminus, was found to promote assimilation of homologous single-stranded DNA into duplex DNA more efficiently than intact recA protein. This difference was not found if *Escherichia coli* single-stranded DNA binding protein was present. The ATPase activity of both intact recA protein and the fragment was identical. The difference in strand assimilation activity cannot be due to differences in single-stranded DNA affinity, since both the fragment and intact proteins bind to single-stranded DNA with nearly identical affinities. However, the fragment was found to bind double-stranded DNA more tightly and to aggregate more extensively than recA protein; both of these properties may be important in strand assimilation. Aggregation of the fragment was extensive in the presence of duplex DNA under the same condition where recA protein did not aggregate. The double-stranded DNA binding of both recA protein and the fragment responds to nucleotide cofactors in the same manner as single-stranded DNA binding, *i.e.* ADP weakens and ATPγS strengthens the association. The missing C-terminal region of recA protein includes a very acidic region that is homologous to other single-stranded DNA binding proteins and which has been implicated in DNA binding modulation. This C-terminal region may serve a similar function in recA protein, possibly inhibiting double-stranded DNA invasion. The possible role of the enhanced double-stranded DNA affinity of the fragment protein in the mechanism of strand assimilation is discussed.

recA protein of *Escherichia coli* is a 37,800-dalton protein that has been shown genetically to be essential for both homologous recombination and induction of the SOS response to DNA damage (for reviews, see Refs. 1-3). Two very different functions of the protein are responsible for these biological activities. The recombination function catalyzes both the renaturation of homologous single strands of DNA to form duplex DNA, and the assimilation of single-stranded DNA into homologous duplex DNA to form joint heteroduplex DNA molecules. Joint molecule formation (*i.e.* strand assimilation), in turn, is followed by branch migration to form fully exchanged DNA strands. SOS induction involves the DNA- and ATP-dependent cleavage of lexA repressor catalyzed by recA protein (for review, see Ref. 4). This same activity is responsible for prophage induction of lambda and P22 through the proteolysis of λ (5) and P22 repressors (6).

In an attempt to understand the mechanistic aspects of the recombinational activities of recA protein, we have taken the approach of looking for limited proteolytic digestion fragments that still retain partial enzymatic activity. By studying the biochemical and physical properties of such protein fragments, two types of complementary information can be obtained: 1) the location within the recA protein primary structure of residues involved in the altered properties, and 2) the relationship between any altered physical properties of the fragment protein and its ability to promote *in vitro* strand exchange. Such studies should provide information on the structural and functional domains within recA protein as well as on mechanistic aspects of recA protein function.

This type of approach has been used successfully to study a number of proteins (7-10). Lonberg *et al.* (10) studied proteolytic fragments of T4 gene 32 protein missing either the C terminus, or both the C and N termini. Dramatic differences in DNA binding affinity, cooperativity, and salt dependence of binding were found and correlated with either one end or the other of gene 32 protein. Using this analysis, a detailed molecular picture of gene 32 protein structure and function was obtained.

The C-terminal regions of a number of DNA binding proteins (11), including recA protein (12), are highly negatively charged. Removal of this region from gene 32 protein of phage T4 has been shown to slightly increase the affinity of the fragment protein for ssDNA,[1] while not affecting site size or cooperativity of binding (10). Despite the fact that the intact gene 32 protein was able to denature synthetic dsDNA molecules, it was kinetically prevented from denaturing natural dsDNA. In contrast, the gene 32 protein fragment was able to melt native dsDNA due to the elimination of this kinetic block to denaturation. Similarly, single-stranded DNA binding protein (SSB) of *E. coli* was also found to be more effective in melting dsDNA after the negatively charged C-terminal

* This work was supported in part by funds from National Institutes of Health Research Grant AI-18987 and from the Earl M. Bane Biomedical Research Fund, and by an American Cancer Society Junior Faculty Research Award (JFRA-70). These results were previously presented at the 29th Annual Meeting of the Biophysical Society, February 24-28, 1985, abstract T-AM-D8. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; SSB, single-stranded DNA binding protein; DTT, dithiothreitol; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl-; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); etheno-M13 ssDNA, single-stranded M13 DNA chemically modified to contain 1,^{6}*N*-etheno-adenylic acid and 3,N^4-etheno-cytidylic acid residues; SDS, sodium dodecyl sulfate; bp, base pair(s).

region was removed by proteolysis (11). Here, we find that removal of the C-terminal region from recA protein results in an increased affinity of the fragment for dsDNA. Because gene 32 protein, SSB, and recA protein all have negatively charged C termini that modulate DNA binding, it appears that this type of C-terminal structure has the general property of affecting DNA affinity. Williams *et al.* (11) have suggested that the function of the negatively charged C termini of ssDNA binding proteins is to attenuate dsDNA denaturation.

In this paper we report studies of a proteolytic fragment of recA protein lacking about 15% of the C terminus. We find that this fragment protein is active in terms of both ATPase activity and strand assimilation activity. Surprisingly, this fragment produces a greater yield of product in the strand assimilation reaction (in the absence of SSB protein) than does intact recA protein, but is identical to intact recA protein in the presence of SSB protein. We find that the affinity of the fragment protein for dsDNA is increased relative to recA protein and that the aggregation properties are also changed, whereas ssDNA binding affinity is not significantly altered. Thus, the increased strand assimilation activity of the fragment relative to recA protein must be the result of the enhanced dsDNA binding or aggregation properties. In the "Discussion," we focus primarily on DNA binding and discuss the role that the acidic C-terminal region might play.

EXPERIMENTAL PROCEDURES

Proteins and DNA—recA protein and etheno-M13 ssDNA were obtained as described by Cox *et al.* (13) and Menetski and Kowalczykowski (14), respectively. Extinction coefficients used were 27,000 M^{-1} cm^{-1} at 280 nm and 7,000 M^{-1} cm^{-1} (15) at 260 nm, respectively.

Both single-stranded phage and supercoiled replicative form DNA from phage M13mp7 were obtained by the method of Messing (16). Linear dsDNA was prepared by digestion with *Eco*RI endonuclease. DNA concentrations were determined using an extinction coefficient of 6500 M^{-1} cm^{-1} for dsDNA and 8780 M^{-1} cm^{-1} for ssDNA at 260 nm.

The proteolytic fragment was produced during storage of a particular recA protein preparation for 6 months at 4 °C; normally, recA protein is completely stable under these conditions. The fragment was purified on ssDNA cellulose using a linear sodium chloride gradient of 0–300 mM in 20 mM Tris-HCl, 0.1 mM DTT, 0.1 mM EDTA, pH 7.5. Fractions containing the fragment were pooled and the protein was precipitated by addition of 0.4 g of ammonium sulfate/ml of solution. The precipitate was collected by centrifugation. The protein was redissolved and dialyzed into 20 mM Tris-HCl, 100 mM NaCl, 0.1 mM DTT, 0.1 mM EDTA, 10% glycerol, pH 7.5, and stored at 4 °C. Weight concentration was determined using the Bio-Rad protein assay based on Coomassie dye binding; dilutions of intact recA protein were used as standards. A molecular weight of 33,000 for the fragment was used to convert from weight to molar concentration.

Protein gel electrophoresis in 10% polyacrylamide SDS-urea gels was performed according to Maizel (17). All protein molecular weight markers except recA protein were obtained from Sigma. Protein gels were either stained by Coomassie Brilliant Blue or silver-stained by the method of Oakley *et al.* (18).

N-terminal Peptide Sequencing—N-terminal sequencing was performed by the method of Gray (19) with some modifications. One-milliliter borosilicate ampules were thoroughly heated in an air-gas flame to destroy any protein traces. Three-quarters of a nanomole of protein in solution was pipetted into each of these tubes. Protein was precipitated using trichloroacetic acid and pelleted. Precipitates were washed once with cold acetone. N-terminal analysis was performed on protein in each tube after none, one, or two rounds of the Edman cycle to determine the first 3 residues. The cycle involves coupling to phenylisothiocyanate, followed by cleavage to release the phenylthiohydantoin-amino acid and expose the next residue as N-terminal. Coupling was performed by dissolving protein in 50 μl of 1% SDS plus 2 μl of *N*-ethylmorpholine, adding 100 μl of 5% phenylisothiocyanate in pyridine and heating at 50 °C for 30 min. Protein was precipitated with cold acetone and the pellet vacuum-dried at 50 °C. Cleavage was accomplished by adding 200 μl of trifluoroacetic acid and placing the samples at 50 °C for 10 min. The trifluoroacetic acid was evaporated under a stream of dry air, and the samples vacuum dried. Thus ends one cycle, with the protein being ready for a second cycle or dansylation. Proteins were dansylated by adding 20 μl of 10% SDS, 200 μl of 0.5 M $NaHCO_3$, pH 8.5, and 100 μl of dansyl chloride in acetone (5 mg/ml), and incubating at 37 °C for 10 min. Protein was precipitated once by 10% trichloroacetic acid (adding extra to neutralize the bicarbonate) and once by acetone. The pellet was vacuum-dried. Hydrolysis was accomplished by sealing ampules containing 100 μl of 5.7 N HCl under aspirator vacuum and heating at 105 °C for 9 h. The hydrolysates were vacuum-dried and dissolved in 5 μl of 50% pyridine. One microliter was spotted on a 5 cm × 5 cm two-sided polyamide thin layer chromatography plate, with dansyl standards spotted on the opposite side. The solvent system of Gray (20) was used. Individual dansyl amino acids were mixed with hydrolysate and run for confirming identification.

The amino acid analysis was done by Dr. Lange of Loyola Medical School.

Functional Assays—ATPase activity was measured either by thin layer chromatography or by an enzyme-coupled spectrophotometric assay. For the chromatographic assay, ATP hydrolysis was measured in 20-μl mixtures containing 0.5 mM ATP, 12 μM heat denatured calf thymus DNA, and 75 μg/ml bovine serum albumin in 20 mM Tris-HCl, 20 mM NaCl, 4 mM $MgCl_2$, 0.1 mM DTT, pH 7.5. To this, 0.2 μCi of [^{3}H]ATP was added. The assay was conducted at 37 °C. Reactions were stopped with excess EDTA, unlabeled ADP, and unlabeled ATP. Nucleotides were separated on polyethylenimine cellulose thin layer chromatography plates (E. Merck, Darmstadt, Federal Republic of Germany) developed with 0.6 M NaH_2PO_4, pH 4.0. ADP and ATP were visible under ultraviolet light, allowing these spots to be cut out and scintillation counted to quantify hydrolysis. Controls without DNA were also run.

The spectrophotometric ATPase assay (21) was carried out at 37 °C as described previously (22–24), with the following differences. The buffer contained 25 mM Tris acetate (pH 7.5), 40 mM sodium acetate, 12 mM magnesium acetate, 1.0 mM DTT, 5% glycerol, 1 mM ATP, 5 mM phosphoenol pyruvate, and 2 mg/ml NADH; in addition, 25 units/ml each of pyruvate kinase and lactate dehydrogenase were present.

The strand assimilation and exchange activities were assayed using the agarose gel assay[2] described by Cox and Lehman (25) as modified by Roman and Kowalczykowski (22). Circular single-stranded and linear duplex M13mp7 DNA were used as substrates for this assay and 12 mM magnesium acetate was present in the reaction mixtures. Reactions were initiated by adding the recA protein or fragment along with SSB protein, if desired, to a mixture of the DNA and other components at 37 °C. DNA products were separated on 0.8% agarose gels containing 0.5 μg/ml ethidium bromide and visualized using ultraviolet irradiation. A photographic negative was scanned densitometrically to quantify the extent of reaction. The results are presented as the fraction of product dsDNA formed, *i.e.* the amount of product formed, divided by the sum of the amounts of product and initial substrate duplex DNA present.

Fluorescence and Light Scattering Titration—All fluorescence and scattering measurements were made on a Perkin-Elmer Spectrofluorometer (model MPF-44E) with a cuvette thermostated to 25 °C. The binding of protein to etheno-M13 ssDNA and salt dissociation of the complex were monitored using the increase in fluorescence associated with protein binding, as described by Menetski and Kowalczykowski (14). The salt titration midpoint is defined as the salt concentration at which 50% of the bound recA protein has dissociated. Excitation and emission wavelengths were 300 and 405 nm, respectively. Evaluation of the cooperativity parameter (26), ω, from the experimental titration data was performed as described by Menetski and Kowalczykowski (14). Protein binding to dsDNA was monitored by changes in ethidium fluorescence. First, ethidium bromide was added to the solution containing duplex DNA. Binding of fragment of recA protein was accompanied by a fluorescence increase. For these experiments 360 nm was used as the excitation wavelength and the emission at 590 nm was observed. Details are given under "Results."

[2] We have recently determined that agarose gel assays run under these conditions do not resolve the gapped circular duplex DNA product molecules (which result from complete DNA strand exchange) from the intermediate joint, or D-loop, DNA molecules (which result from strand assimilation) (J. P. Menetski and S. C. Kowalczykowski, manuscript in preparation). Consequently, we are unable to distinguish between those two DNA products here.

Intrinsic tryptophan fluorescence was observed by excitation at 250 nm, and scanning from 290 to 420 nm to define the broad emission peak at 340 nm.

Scattering was measured as described previously (22) by setting the excitation and emission wavelengths of the fluorimeter to 350 nm.

RESULTS

SDS-urea electrophoretic analysis of a particular preparation of recA protein stored at 4 °C for 6 months showed that nearly quantitative proteolytic conversion had occurred to yield a well-defined polypeptide. Preliminary experiments demonstrated that the major resulting fragment was likely to be at least partially active in DNA strand assimilation and ATP hydrolysis. For this reason, we felt that this fragment would be a good candidate for study, and proceeded to purify it.

The fragment was found to bind to a single-stranded DNA-cellulose column and eluted from the DNA cellulose at 125 mM NaCl as a single peak. Protein from the peak produced a single band on SDS-urea gels (>99%); no other bands were detectable on an overloaded, silver-stained gel. Comparison of this band to ovalbumin (43 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), and recA protein (37.8 kDa), yielded a size of 33 kDa for the fragment. Dansyl Edman microsequencing of the fragment established that the three N-terminal residues were Ala-Ile-Asx, the same as for intact recA protein. This sequence of 3 amino acid residues is unique within intact recA protein and occurs only at the N terminus. The validity of this determination was confirmed by sequencing the terminal 3 residues of intact recA protein. In both cases, the identification of each dansyl amino acid residue was verified by co-migration of the unknown residue with a derivatized amino acid standard. The amino acid analysis of the fragment protein is presented in Table I. The total number of residues found was 287 ± 10, adding one tryptophan to compensate for destruction during hydrolysis. Also shown in Table I is the expected amino acid composition of a fragment of recA protein lacking 45–62 C-terminal residues, as well as that of intact recA protein (12). The observed amino acid analysis is consistent with that expected for a fragment which is lacking the C-terminal region. All of these results taken together demonstrate that the fragment is lacking approximately 50 residues from the C terminus.

TABLE I
Amino acid analysis

	Found	Expected	
		Fragment	recA
Alanine	31.2	33–36	38
Arginine	11.9	13	14
Asparagine/aspartic acid	27.3	25–26	35
Cysteic acid	2.9	3	3
Glycine	30.9	31–34	35
Glutamine/glutamic acid	35.7	34–36	43
Histidine	2.9	2	2
Isoleucine	21.8	25–26	27
Leucine	26.7	27	31
Lysine	22.3	20–23	27
Metionine sulfoxide	8.8	9	9
Phenylalanine	(8)	8	10
Proline	7.5	7	10
Serine	13.7	15–16	20
Threonine	10.7	13–14	17
Tryptophan	N.D.	1	2
Tyrosine	6.8	5–7	7
Valine	17.2	17	22
Total	287.3	290–307	352

The location of the cleavage site was also confirmed by the fortuitous placement of the 2 tryptophan residues in the primary sequence of recA protein at positions 290 and 308. Because the fluorescent quantum yield of tryptophan is much greater than that of tyrosine, and assuming that the fluorescent yield is not significantly altered by structural changes associated with the proteolysis, one would expect the fluorescence intensity to be reduced by approximately one half if the cleavage site was between these 2 residues. To explore this possibility, fluorescence measurements on solutions of recA protein and fragment of equal molarity were performed. The tryptophan fluorescence peak of the fragment (340 nm) was found to be about 45% of that of recA protein (not shown). This result confirms identification of the cleavage site as between residues 290 and 308.

Attempts to further define the location of the C-terminal cleavage site through direct determination of the C-terminal amino acid residues by digestion with carboxypeptidase A, B, or Y were unsuccessful. In addition, efforts to produce additional amounts of this fragment protein by partial proteolytic digestion using either trypsin, chymotrypsin, or *Staphylococcus aureus* V8 protease failed to produce this fragment protein in the high yield obtained fortuitously; in general, many partial proteolytic fragments were produced with relatively poor yield.

ATPase Activity—One of the characteristic enzymatic properties of recA protein is its DNA-dependent ATPase activity. The ability of the fragment to hydrolyze ATP in a ssDNA-dependent reaction was compared to intact recA protein and the comparison is shown in Fig. 1. The reaction was dependent on DNA and the rates of ATP hydrolysis were identical for the two proteins. This was also true if a limiting concentration of ssM13 DNA was used (not shown), implying that the ability of both the fragment and intact proteins to utilize ssDNA was identical (23).

When the dsDNA-dependent ATPase activity was examined, a distinct difference was obtained (Fig. 2). The intact recA protein showed a typical time course consisting of both lag and growth phases (24), whereas the fragment recA protein showed no detectable lag phase and only a linear rate of ATP hydrolysis. The rate of ATP hydrolysis for the fragment protein is 4.74 μM/min and the terminal rate observed for the intact protein is 2.47 μM/min. Thus, the properties of the fragment protein are such that the rate-limiting process which corresponds to the lag phase of the dsDNA-dependent ATP hydrolysis reaction is overcome.

Strand Exchange Activity—An activity of recA protein that is reflective of its viability in genetic recombination is its ability to promote the assimilation and complete exchange of homologous ssDNA into dsDNA. This activity can be assayed

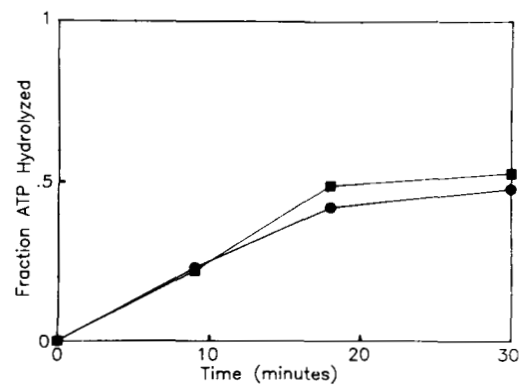

FIG. 1. **Single-stranded DNA-dependent ATPase activity.** ●, intact recA protein; ■, fragment protein; 1.3 μM each at 37 °C.

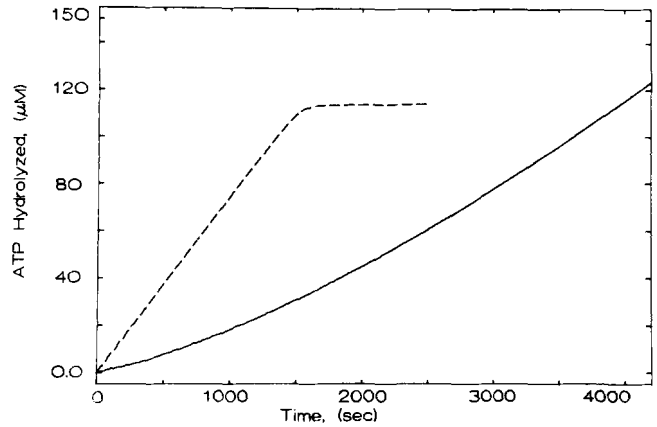

FIG. 2. **Double-stranded DNA-dependent ATPase activity.** Reactions were carried out as described under "Experimental Procedures," with 4.2 μM linear M13 dsDNA and 1.5 μM protein. *Solid line*, recA protein; *dashed line*, fragment protein.

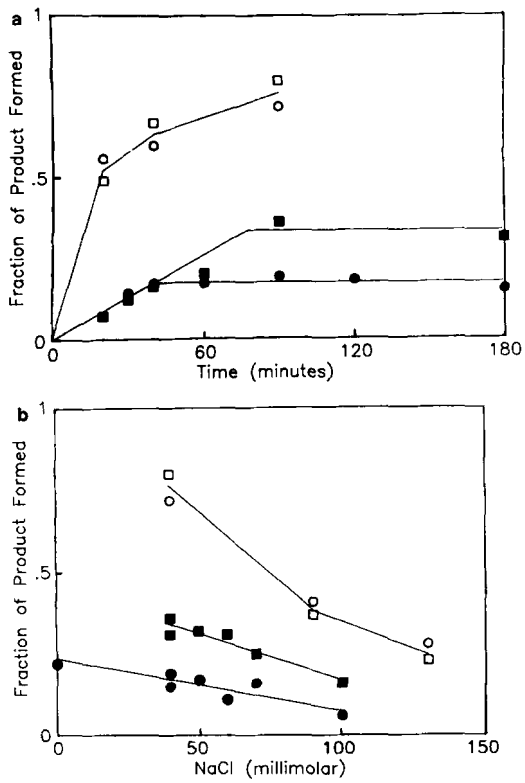

FIG. 3. **Strand assimilation activity of intact and fragment recA proteins.** In *a*, the buffer is 25 mM Tris acetate, 40 mM NaCl, 12 mM magnesium acetate, 1 mM DTT, 5% glycerol, pH 7.5; 37 °C. Same conditions are in *b*, but for varying sodium chloride concentrations. ●, 6 μM recA protein; ■, 6 μM fragment; ○, 6 μM recA protein, 0.9 μM SSB; □, 6 μM fragment, 0.9 μM SSB.

in vitro by using the agarose gel assay described by Cox and Lehman (25). The results of such an assay for both intact recA protein and the fragment are shown in Fig. 3*a*. The data show that, in the absence of SSB protein, the fragment was much more active than recA protein. At 40 mM NaCl there is approximately a 2-fold difference in the final amount of heteroduplex DNA formed. This difference in activity is clearly reflected in the extent of reaction, but not necessarily in initial kinetics of the reaction. The rates of product formation are indistinguishable for nearly the first 40 min of the reaction, but difficulty in measuring the small quantities of product formed preclude conclusions about relative initial rates. (Due to precipitation of the fragment protein under low NaCl concentrations, it was not possible to measure the rate of this reaction under conditions that support a greater yield of products, *i.e.* in the absence of NaCl). As the sodium chloride concentration is increased to 100 mM, the total amount of product formed decreases for both recA protein and fragment. These data, presented in Fig. 3*b*, show that intact recA protein catalyzes the formation of only about one-half the amount of product formed by the fragment protein at all salt concentrations, and that the relative salt sensitivity of these two proteins is similar.

The effect of SSB on the reaction is also shown in Fig. 3, *a* *b*. Interestingly, if SSB is present, no significant differences in the strand exchange reaction promoted by recA protein or the fragment are observed, with both the rate and extent of reaction being much greater in the presence of SSB. In addition, increasing salt concentration inhibits both reactions to the same extent. The differences observed for the fragment and intact proteins in these functional assays prompted us to search for changes in DNA binding or aggregation properties which might account for these differences.

Single-stranded DNA Binding—By using a fluorescent derivative of M13 ssDNA, etheno-M13 ssDNA, Menetski and Kowalczykowski (11) explored binding of recA protein to ssDNA in the presence and absence of nucleotide cofactors and under salt conditions where cooperativity of binding could be determined. It was demonstrated that the binding of recA protein to single-stranded DNA is sensitive to salt concentration, and that relative binding affinity can be determined by measuring the salt concentration required to dissociate a preformed recA protein-DNA complex. The stability of the recA protein-ssDNA complex was shown to decrease upon ADP binding and increase with ATPγS binding.

Binding of recA protein and the fragment to etheno-M13 DNA in the absence of any nucleotide cofactor is shown in Fig. 4*a*. The binding site size of the fragment is the same as that of recA protein, 8 ± 1 bases per protein monomer. The single-stranded DNA binding affinity of the fragment protein relative to intact recA protein can be assessed by comparing values for the salt titration midpoints. The midpoint of the salt dissociation curve (Fig. 4*b*) was found to be only slightly lower for the fragment (270 mM NaCl), when compared to recA protein (285 mM), demonstrating that single-stranded DNA binding is slightly weakened by the absence of the C-terminal region. This result indicates that the increased strand assimilation activity of the fragment protein relative to the intact protein is not simply a consequence of an increased affinity of the fragment for single-stranded DNA.

To determine whether the modulation of single-stranded DNA binding affinity by nucleotide cofactors is different for the fragment protein, the effect of nucleotides on the salt-titration midpoint of the different protein-DNA complexes was investigated. ADP was found to lower the salt-titration midpoint of both fragment and recA protein to 170 mM NaCl (Table II). The presence of ATPγS was found to raise the affinity to such a level that a salt concentration of up to 1.2 M did not disrupt the binding. These results demonstrate that the nucleotide binding sites are intact and are still capable of the dramatic modulation of protein-DNA interactions previously reported for recA protein (14). In addition, they demonstrate that differences in nucleotide modulation of ssDNA affinity are not responsible for the increased activity of the fragment protein.

The binding of the recA fragment protein to etheno-M13 ssDNA was also measured in 270 mM NaCl in the absence of nucleotide cofactors. At this salt concentration the binding is

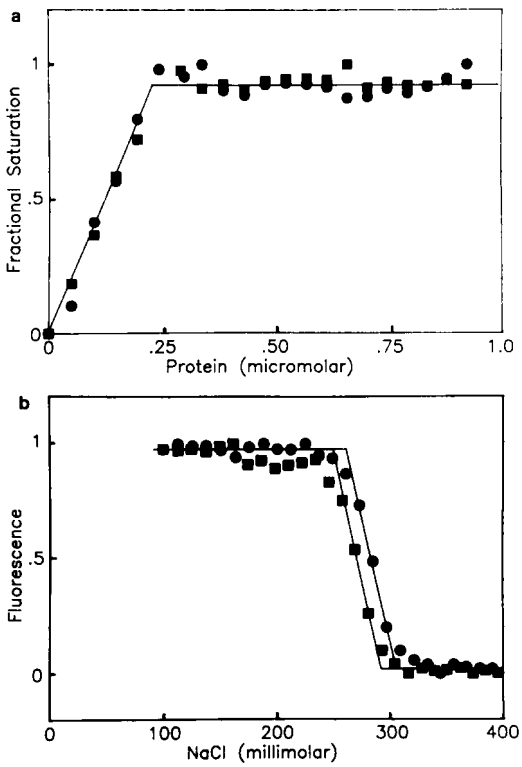

FIG. 4. **Protein binding to etheno-M13 ssDNA.** *a* shows protein titration in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT and containing 1.1 μM etheno-M13 ssDNA; 25 °C ●, recA protein; ■, fragment. Salt dissociation of protein-etheno M13 ssDNA complexes is shown in *b*. Dissociation effected by addition of sodium chloride to cuvettes at the end of protein binding measurements shown in *a*.

TABLE II

Salt dissociation midpoints for protein-DNA complexes

All in 20 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM DTT at 25 °C.

	ssDNA[a]	dsDNA[b]
RecA		
No cofactor	285 mM NaCl	10 mM NaCl
+100 μM ADP	170 mM NaCl	No binding
+100 μM ATPγS	>1.2 M NaCl	>80 mM NaCl
Fragment		
No cofactor	270 mM NaCl	40 mM NaCl
+100 μM ADP	170 mM NaCl	22 mM NaCl
+100 μM ATPγS	>1.2 M NaCl	>80 mM NaCl

[a] All solutions contained 1.1 μM etheno-M13 ssDNA and 1 μM either recA or fragment protein.

[b] Values obtained without cofactor are taken from data in Fig. 4*b*; ADP and ATPγS values were obtained from solutions with 1.8 μM (bp) replicative form M13 DNA and 0.28 μM recA or fragment protein.

weaker, allowing cooperativity of DNA binding to be determined. The results are shown in Fig. 5. the sigmoidicity that is characteristic of cooperative binding is apparent in this curve. Binding analysis according to McGhee and von Hippel (26), yielded a cooperativity parameter (ω) of 25–50. This agrees with a value of 50 found by Menetski and Kowalczykowski (14) for recA protein under similar conditions. This result demonstrates that the protein-protein interactions required for cooperative binding are not disrupted by the absence of the C terminus.

Double-stranded DNA Binding—To measure protein binding to dsDNA, we monitored the effect of added recA protein on the fluorescence of ethidium bromide which was intercalated into the duplex DNA. We found that the fluorescence signal increased by about 10% upon addition of either recA protein or the fragment, suggesting that there was either increased ethidium binding or increased fluorescence quantum yield due to the formation of a recA protein-duplex DNA complex. We found distinct and reproducible break points in the protein titration curves, as one would expect in the case of protein saturation of the nucleic acid. Binding to supercoiled replicative form DNA is presented in Fig. 6*a*. This data, which are typical of data obtained for linear and supercoiled DNA, show break points that suggest an apparent site size of 10 base pairs for both proteins. Apparent site size values of 8–12 base pairs were found for both linear and supercoiled DNA, with no distinguishable trend apparent in site size differences for the two types of DNA or the two proteins.

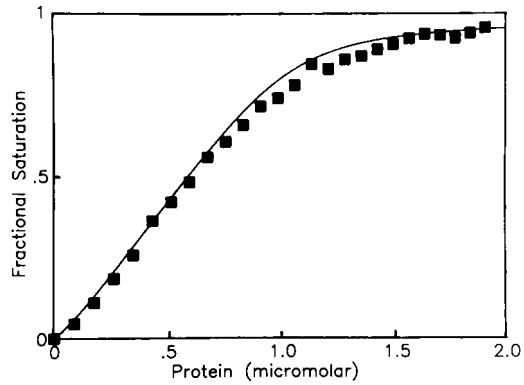

FIG. 5. **RecA fragment protein binding to etheno-M13 ssDNA in high salt.** The buffer is 20 mM Tris-HCl (pH 7.5), 270 mM NaCl, 4 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM DTT and contains 4.3 μM etheno-M13 ssDNNA; 25 °C. *Curve* shows theoretical binding curve assuming a value for the cooperativity (ω) of 25. The apparent affinity, K_ω, determined from the midpoint of the titration data is 5.50 μM^{-1}, yielding a value for K of 0.22 μM^{-1}.

We also found that the fluorescence of the protein-dsDNA-ethidium bromide complex returned to the initial value observed for the dsDNA-ethidium bromide complex (*i.e.* that obtained before the addition of protein) upon addition of sodium chloride. The salt concentration required for half-dissociation was characteristic of the type of protein complex present. The salt dissociation data are presented in Fig. 6*b*. These data show a clear and significant difference in salt dissociation midpoint values between recA protein and the proteolytic fragment. recA protein starts to dissociate upon the first addition of NaCl, with the midpoint occurring at about 10 mM NaCl. The fragment has a salt dissociation midpoint of about 40–45 mM NaCl, indicating that the fragment-duplex DNA complex is more stable to dissociation by salt. The results are the same with supercoiled DNA. In the presence of ATPγS (Table II), there is no fluoresence decrease for either protein-DNA complex until about 80 mM NaCl. This, unfortunately, is the salt concentration where the ethidium-dsDNA complex alone starts to show a decrease in fluorescence. Although this precludes a quantitative statement about binding, it is clear that both proteins form complexes with dsDNA in the presence of ATPγS that are more resistant to dissociation by salt than are the complexes formed in the absence of ATPγS. This is the same effect found for ssDNA binding (14). ADP decreases the stability of both protein-dsDNA complexes when compared with the stability in the absence of nucleotide (Table II). The addition of 100 μM ADP results in the dissociation of the intact recA protein supercoiled dsDNA complex without the need for added sodium chloride. The same concentration of ADP lowers the salt

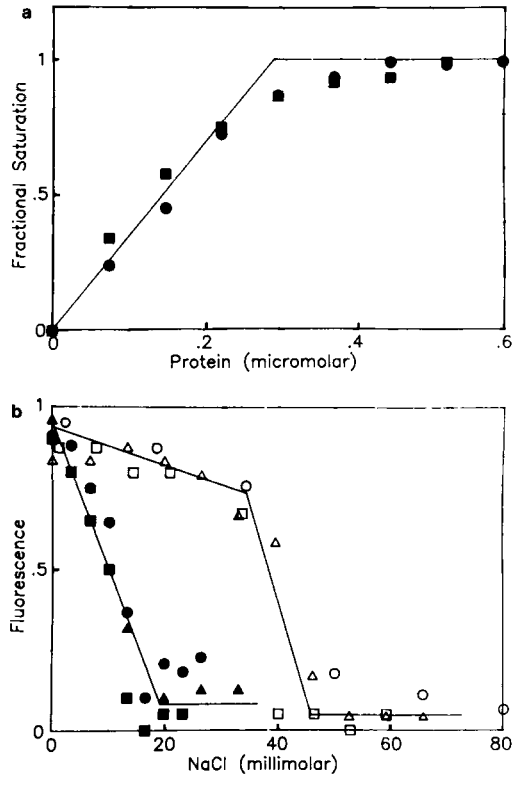

FIG. 6. **recA and fragment protein binding to M13 dsDNA.** In *a*, the buffer is 20 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM DTT, and contains 2.3 μM (in base pairs) replicative form M13 DNA. ●, recA protein; ■, fragment. Salt dissociation of protein-dsDNA complexes is shown in *b*. Dissociation effected by addition of sodium chloride. ●, 0.40 μM recA protein, 4.47 μM (bp) replicative form M13 DNA; ■, 0.28 μM recA protein, 1.63 μM (bp) linear M13 dsDNA; ▲, 0.48 μM recA protein, 3.47 μM (bp) linear M13 dsDNA. ○, 0.34 μM fragment, 2.3 μM (bp) replicative form M13 DNA; □, 0.18 μM fragment, 0.72 μM (bp) linear M13 dsDNA; △, 0.24 μM fragment, 1.62 μM (bp) linear M13 dsDNA.

dissociation midpoint of the fragment protein-supercoiled dsDNA complex to 22 mM NaCl. Thus, it is clear that, in the presence or absence of ADP, the stability of the complex formed between the fragment recA protein and duplex DNA is greater than that of the intact protein-dsDNA complex. This demonstrates that the fragment has a greater affinity for duplex DNA than does the intact protein. Furthermore, the effect of the two nucleotides on dsDNA binding affinity is dramatic and the same as their effect on ssDNA binding affinity.

Although the ethidium bromide method is useful for examining relative binding strengths of the protein for dsDNA, it is possible that the presence of the ethidium bromide shifts the measured affinities to higher or lower values than would be found in its absence. To test this possibility, we determined the salt titration midpoint of recA protein supercoiled dsDNA complexes under a variety of conditions. These results are shown in Table III. Over a 50-fold increase in ethidium bromide concentration, we found a 2-fold increase in salt titration midpoint of protein-DNA complex from 8 to 16 mM NaCl. Therefore, all dsDNA binding experiments reported in this paper were conducted with 1.70 μM ethidium bromide to avoid differences in protein affinity due solely to changes in ethidium bromide concentration. A further complication in the quantitative interpretation of the duplex DNA binding data is the presence of a slow approach to equilibrium of the fluorescence signal (not shown). This effect is qualitatively similar to the slow lag phase that is observed in the dsDNA-

TABLE III

Dependence of the salt titration midpoint for recA protein-dsDNA complexes on the ethidium bromide concentration

All in 20 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM DTT at 25 °C.

[Ethidium bromide]	[DNA] (bp)	[recA protein]	[NaCl] midpoint
	μM		*MM*
0.10	2.8	0.25	8
1.19	2.8	0.25	10
0.68	9.0	2	10
1.70	9.0	2	11
5.10	9.0	2	16

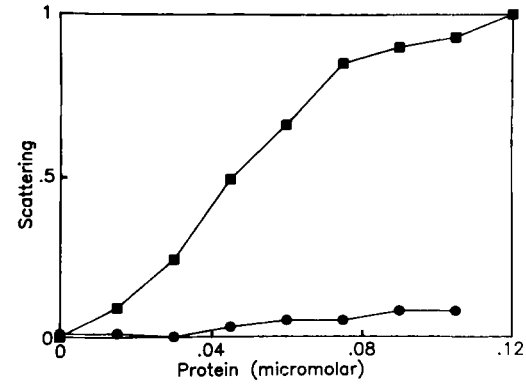

FIG. 7. **Scattering of protein-dsDNA complexes.** The buffer is 20 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM DTT and contains 0.45 μM (bp) replicative form M13 DNA; 25 °C. ●, recA; ■, fragment.

dependent ATPase activity (22, 24, 27) and makes interpretation of the site size difficult. The apparent site size of 8–12 base pairs is slightly larger than the values reported in the literature which range from 2 to 8.5 base pairs per monomer of recA protein (24, 27–31). These questions do not, however, interfere with use of the salt dissociation data to determine the relative affinities of the two proteins for dsDNA.

The salt concentration required for complex dissociation increases with increasing ethidium concentration, as shown in Table III. This suggests positive, cooperative linkage between ethidium and recA protein binding, *i.e.* binding of one species promotes binding of the other. This is supported by the increase in fluorescence upon protein binding, which suggests increased ethidium binding to the DNA. The source of this cooperative effect is likely to be due to the similar changes in DNA structure that ethidium and recA protein induce; both have been shown to unwind and elongate dsDNA upon binding (32–34).

Aggregation—During isolation, the proteolytic recA protein fragment precipitated if stored in 20 mM Tris-HCl, 10% glycerol, 0.1 mM EDTA, 0.1 mM DTT, pH 7.5, but remained in solution if 100 mM sodium chloride was added. Because recA protein aggregation reflects protein-protein interactions that might be involved in strand exchange (35), we explored these properties using light scattering.

Dramatic differences in protein aggregation were found in the presence of dsDNA. Under the same buffer conditions used to measure protein binding to dsDNA, a dramatic increase in intensity of scattered light was found to accompany fragment binding to replicative form M13 DNA. As shown in Fig. 7, this scattering increase did not occur with recA protein. The same results were found with linear dsDNA. Similar scattering effects have been observed for the intact protein only at low pH values (27). (This increase in light scattering is different from the DNA-independent increase observed

upon increasing the Mg^{2+} ion concentration (36); the proteolytic fragment of recA protein (at 0.5 μM) did not show a Mg^{2+}-induced aggregation up to 18 mM $MgCl_2$, although the initial scattering in the absence of Mg^{2+} was slightly higher (not shown).) The scattering signal obtained in Fig. 7 was very salt-sensitive. A slow time-dependent decrease in intensity was initiated by raising the sodium chloride concentration from 9 to 13 mM. This process occurred over approximately a 1-h time span. This is well below the 40–45 mM NaCl required to dissociate the fragment protein-dsDNA complex and follows very different kinetics than the rapid salt-induced dissociation of the protein-DNA complex. For these reasons, the scattering is thought to be due to the aggregation of protein-dsDNA complexes rather than to the binding of protein to DNA.

DISCUSSION

We have described the properties of a proteolytic fragment of recA protein that was found to be missing about 15% (≈50 amino acid residues) from the C terminus. This fragment protein is still functional with regard to its ATPase activity and strand assimilation activity. Surprisingly, we find that the fragment protein has increased strand assimilation activity compared to intact recA protein; however, this difference disappears in the presence of SSB. These results indicate that 15% of the C terminus of recA protein is not essential to the *in vitro* function of the protein. Studies carried out by Yarranton and Sedgwick (37) on a set of C-terminal truncated recA genes showed that deletion of as little as 21% of the carboxyl end of the protein results in a non-functional protein *in vivo*. Their results, taken together with those presented in this paper, lead to one of two conclusions. The simplest conclusion is that the region of recA protein located approximately between amino acid residues 280 (21%) and 300 (15%) is critical to recA protein function; this would predict that the 21%-truncated protein would be nonfunctional *in vitro*. Alternatively, it is also possible that the C terminus of the protein is, in fact, important *in vivo* but that this functionality is not manifest *in vitro* due to limitations of the *in vitro* assays. A number of recA mutations have been sequenced recently and none map beyond amino acid residue 301 (38, 39). This observation suggests that either this region is not important to recA protein function, or simply that an insufficient number of mutants have been sequenced. Additional studies, in progress, will be required to discriminate between the above possibilities.

Based on *in vivo* mutational studies (38, 39) it was concluded that the N terminus of recA protein is the site of single-stranded DNA binding and is also responsible for protein-protein interactions. Our direct DNA binding studies do not contradict these conclusions, in that we have shown the fragment protein binds to single-stranded DNA with virtually the same affinity and degree of cooperativity as the intact recA protein. Thus, we conclude that the ssDNA binding site and the site of protein-protein interactions responsible for cooperative binding do not reside in the C-terminal region.

In an attempt to understand the molecular mechanism responsible for the increased strand assimilation activity caused by removal of the C terminus, we examined several physical properties of the fragment protein. Quantitative DNA binding studies showed that the single-stranded DNA affinity is relatively unchanged, as judged by the salt-titration midpoint. In addition, both the intact and fragment proteins showed identical ssDNA-dependent ATPase activity. Therefore, these properties are not likely to be the cause of the enhanced activity. However, three properties of the fragment protein were found to be significantly different from those of the intact recA protein: 1) the fragment protein showed no lag and a higher rate of ATP hydrolysis in the dsDNA-dependent ATPase activity; 2) the duplex DNA binding affinity as measured by the salt titration midpoint was much higher; and 3) the fragment showed an increased propensity to aggregate in the presence of dsDNA. Thus, changes in any one or all of these properties could be the cause of the increased activity. This suggests that the double-stranded DNA binding differences are the likely source of the increased strand assimilation yield by the fragment. Consistent with this interpretation, recent studies suggest a relationship between duplex DNA-dependent properties and the yield of product in a strand exchange reaction (22).

Biochemical studies of a C-terminal deletion fragment of the recA protein purified from *Proteus mirabilis* have been also described (40). That fragment protein is 36 kDa in size (*i.e.* lacking about 25 amino acid residues) and possesses properties that are very similar to those described here for the 33-kDa fragment derived from *E. coli*. Both fragment proteins possess similar ssDNA-dependent ATPase and ssDNA binding activities when compared with their respective intact proteins. In addition, both fragment proteins display the same altered activities relative to their intact protein counterparts, namely, enhanced binding to dsDNA, aggregation upon binding to dsDNA, and increased product formation in the strand assimilation reaction when SSB protein is absent. Thus, despite the differences in fragment size and bacterial origin, these two fragment recA proteins appear to behave rather similarly, further implicating the importance of dsDNA-dependent properties in the strand assimilation process and the involvement of the C terminus in these activities.

The specific molecular explanation for the increased product formation in the DNA strand assimilation reaction is unknown. However, since the ssDNA-dependent properties (ATPase activity and DNA binding) are unaltered, an enhanced ability to utilize ssDNA within secondary structures cannot be responsible for the increase. Elsewhere, we have presented evidence for the importance of dsDNA-dependent processes (*e.g.* ATPase activity) in DNA heteroduplex formation (2, 22, 24). This general conclusion also applies to the fragment recA protein. The kinetic mechanism of dsDNA-dependent ATPase activity was shown to proceed by a two-step mechanism: a slow, rate-limiting nucleation step, followed by a rapid growth step. Since the fragment protein shows essentially no lag in its dsDNA-dependent ATPase activity and an enhanced dsDNA binding activity, this implies that these two properties are in some way related. The specific nature of this relationship is uncertain; however, there are two limiting possibilities. The first is that, due to the enhanced dsDNA binding ability of the fragment protein, the amount of dsDNA complex formation is increased (to form more of the "closed" complex (24)) resulting in a much greater probability of dsDNA invasion (stoichometric saturation of M13 dsDNA is not observed with intact recA protein[3]). The second possibility is that the fragment protein is altered in such a way that it is kinetically more capable than the intact protein at utilizing dsDNA as a substrate for ATP hydrolysis. This intrinsic kinetic difference could be due to alteration in a property of the protein alone (*e.g.* self-association) or in a property of the protein-dsDNA complex (*e.g.* an enhanced ability to invade or "trap" transiently open dsDNA). Further detailed studies on the mechanism of the dsDNA-dependent

[3] S. C. Kowalczykowski and R. A. Krupp, unpublished observations.

ATPase activity will be required to discriminate between such possibilities.

Finally, the function of the C-terminal region of recA protein can be addressed. It is interesting to note that this region of the protein is rich in acidic amino acid residues. The presence of an acidic tail in other single-stranded DNA binding proteins (SSB protein, gene 32 protein, T7 ssDNA binding protein, and fd gene 5 protein) has been previously noted by Williams *et al.* (11). It has been found that proteolytic removal of this acidic tail results in altered duplex DNA destabilization properties. Removal of the tail from SSB protein results in a fragment protein with enhanced helix-destabilization ability. In the case of gene 32 protein, removal of this region results in a protein fragment that no longer displays a kinetic inhibition to duplex DNA denaturation, and yet the ssDNA binding properties are essentially unaltered (10, 41). We have obtained qualitatively similar results for recA protein. Our results suggest a similar function (although not identical) for the acidic tail of recA protein, with the increased strand assimilation activity of the protein fragment being due to the lack of this tail. We have shown that nucleotide cofactors have the same relative effects on dsDNA binding that they do on ssDNA binding; *i.e.* ATPγS increases the affinity and ADP decreases the affinity of recA protein for both types of DNA. The fact that both the fragment and intact proteins have similar ssDNA binding affinities but different dsDNA binding affinities suggests that the C-terminal tail somehow interferes with dsDNA binding (*e.g.* perhaps this region of the protein interferes sterically or electrostatically with the opposite strand of the DNA duplex). In addition, the fragment protein shows no lag in the dsDNA-dependent ATPase activity. This implies that the C terminus is involved in a protein structural transition or change in aggregation state which also affects the duration of rate-limiting lag phase normally observed with the intact protein. This lag phase could represent the formation of an appropriately sized cluster of proteins bound to dsDNA and required for nucleation as previously proposed (2, 24, 42). Additional studies will be required to delineate the exact function of this C-terminal region of recA protein.

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